

Appendix H

Toxicity Testing Report



RESULTS OF TOXICITY TESTING WITH
Hyalella azteca AND *Chironomus riparius* ON SEDIMENT SAMPLES
FROM THE SUPERIOR WATERFRONT CHARACTERIZATION
ST. LOUIS RIVER AREA AND BAY AREA OF CONCERN, MICHIGAN

Prepared for:

U.S. Environmental Protection Agency
Region 5
77 West Jackson Boulevard
Chicago, Illinois 60604-3507

Prepared by:

EA Engineering, Science, and Technology, Inc., PBC
231 Schilling Circle
Hunt Valley, Maryland 21031
For questions, please contact Michael Chanov
ph: 410-584-7000

Results relate only to the items tested or to the samples as received by the laboratory.

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This report contains 17 pages plus 4 attachments.

Wayne L. McCulloch
Laboratory Director

28 October 2015

Date

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1. INTRODUCTION

At the request of U.S. Environmental Protection Agency (US EPA), EA Engineering, Science, and Technology performed sediment toxicity tests on six sediment samples from the Superior waterfront within the Wisconsin portions of St. Louis Bay and Superior Bay. The sediment samples were evaluated using 10-day survival and growth toxicity tests with the freshwater midge *Chironomous riparius*. Additionally, 28-day survival, growth and reproduction toxicity tests were conducted with the freshwater amphipod *Hyaella azteca*. The objective of the toxicity testing was to evaluate the toxicity of each of the site sediment samples based on the survival, growth or reproduction (*H. azteca*) of the test organisms, as compared to control sample.

2. METHODS AND MATERIALS

2.1 SAMPLE DESCRIPTIONS

Six sediment samples were collected by EA Personnel. The samples were packed on wet ice and shipped via overnight courier to EA's Ecotoxicology Laboratory in Hunt Valley, Maryland. Upon receipt at EA, the samples were visually inspected, compared against the chain-of-custody record, and assigned EA laboratory accession numbers. A copy of the chain-of-custody record is included in Attachment I. Table 1 summarizes the collection and receipt data for the six site sediments. When not being processed for testing, the samples were stored in the dark at 4°C.

2.2 CONTROL SEDIMENT

A control sediment was initiated with the site sediments. The control sediment was a natural sediment from Pretty Boy Reservoir, Maryland which has been utilized previously in amphipod and midge sediment toxicity testing.

2.3 LABORATORY WATER

Dechlorinated tap water was used to culture the *H. azteca*, and as overlying water for the *H. azteca* 28-day survival, growth and reproduction test and *C. riparius* 10-day survival and growth tests. The source of the water was the City of Baltimore municipal water system. Upon entering the laboratory, the water passed through a high-capacity, activated-carbon filtration system to remove any possible contaminants such as chlorine and trace organic compounds. This water source has proven safe for aquatic organism toxicity testing at EA as evidenced by maintenance of the multigeneration *Hyalella azteca* and fathead minnow cultures with no evident loss of fecundity.

2.4 TEST ORGANISMS

The midges (*Chironomus riparius*) lot CH-057 were obtained from Environmental Consulting and Testing (Superior, WI). Upon receipt at EA on 28 August 2015, the organisms were gradually acclimated to laboratory water at 23°C. Second to third-instar larvae were used in the toxicity testing.

The amphipods (*Hyalella azteca*) were obtained from EA's in-house cultures. The amphipods were cultured at 23°C in 10-gallon glass aquaria with a substrate of maple (*Acer* sp.) leaves and overlying water of dechlorinated municipal tap water. Prior to introduction into the aquaria, the leaves were pre-soaked to remove tannins. Organisms were 7 to 8 days old for testing, and were acclimated to the testing temperature of 23°C during the holding period.

2.5 TOXICITY TEST OPERATIONS AND PERFORMANCE

Toxicity testing was conducted in accordance with US EPA guidance (US EPA 2000), and test methodologies followed EA's standard toxicity testing protocols (EA 2013). The sediment samples were evaluated for toxicity with *H. azteca* and *C. riparius* and the test results comply with current NELAC standards where applicable.

2.5.1 Survival and Growth Toxicity Test with *Chironomous riparius*

A 10-day midge (*C. riparius*) toxicity test was conducted on the composited site sediment samples. The test chambers were 300-ml lipless glass beakers each containing 100 ml of sediment and 175 ml of overlying water (lab water). The tests were performed with eight replicates per sediment. The sediments and overlying water were added to the chambers 24 hours prior to introduction of the test organisms. The beakers were left undisturbed overnight to allow any suspended sediment particles in the water column to settle. Overlying water in the test chambers was renewed once prior to test initiation in order to remove any residual ammonia prior to introduction of test organisms. The introduction of the test organisms to the test chambers on

the following day marked the initiation of the toxicity tests. Ten organisms were randomly introduced into each replicate beaker for a total of 80 organisms per sediment. The test chambers were placed in a water bath to maintain temperatures at a target range of $23\pm1^{\circ}\text{C}$, with a 16-hour light/8-hour dark photoperiod.

The *C. riparius* were fed 1.5 ml per replicate of a 4 g/L slurry of Tetramin flake food daily. The overlying water in the exposure chambers was renewed a minimum of twice daily using a water delivery system (Zumwalt et al. 1994). Fresh overlying water was slowly added to each replicate, displacing the water already in the beaker to flow out through a notch cut into the top of the beaker. The notch was sealed with fine mesh screen to prevent loss of organisms during the renewal process.

For midge toxicity testing, water quality parameters of temperature, pH, dissolved oxygen, and conductivity were recorded daily on the overlying water in one replicate of each sediment. Composite samples of the overlying water of each sediment were also analyzed for alkalinity, hardness, conductivity and ammonia at test initiation and test termination.

At the end of the 10-day exposure period, the surviving organisms from each replicate were retrieved from the sediment. The number of surviving organisms from each replicate was recorded, and the surviving organisms from each replicate were placed in a dried, pre-weighed ceramic crucible, and placed in a drying oven at 100°C for 24 hours. The crucibles were then removed from the oven, placed in a desiccator to cool, and weighed. The dry weight of the surviving organisms in each replicate was determined by subtracting the weight of the empty crucible from the weight of the crucible plus dried organisms. The mean dry weight per organism was obtained by dividing the total organism dry weight per replicate by the number of surviving organisms per replicate.

The ash-free dry weight was determined for the midges by placing the crucibles with oven-dried organisms in a muffle furnace at 550°C for two hours, then weighing the crucibles with organisms following an appropriate cooling period. For each replicate, the weight of the crucible

with furnace-dried organisms was subtracted from the weight of the crucible with oven-dried organisms, yielding a total organism ash-free dry weight. A mean ash-free dry weight per organism was obtained by dividing the total organism ash-free dry weight per replicate by the number of surviving organisms per replicate.

The survival and growth results from the *C. riparius* toxicity tests were statistically analyzed according to US EPA guidance (US EPA 2000) to determine if the site sediments were significantly different ($p=0.05$) from the control sediment. If the data were normally distributed, then a t-Test was performed to detect statistically significant differences between test sediments and the control sediment. If the data distribution was non-normal, then a Wilcoxon Two-Sample Test was used to compare the group means. Shapiro-Wilk's Test was used to determine if the data were normally distributed, and the F-Test was used to test for homogeneity of variance.

Table 2 presents a summary of the water quality measurements recorded during the *C. riparius* toxicity testing, and Table 3 summarizes the results of the *C. riparius* tests. Copies of the original data sheets and statistical analyses for the toxicity test are provided in Attachment II.

2.5.2 Survival, Growth and Reproduction Toxicity Test with *Hyaella azteca*

A 28-day amphipod (*H. azteca*) toxicity test was conducted on the composited site sediment samples. The test chambers were 300-ml lipless glass beakers each containing 100 ml of sediment and a minimum of 175 ml of overlying water. The tests were performed with eight replicates per sediment sample. The sediment and overlying water were added to the chambers 24 hours prior to introduction of the test organisms in order to allow any suspended sediment particles in the water column to settle. Overlying water in the test chambers was renewed once prior to test initiation in order to remove any residual ammonia prior to introduction of test organisms. The introduction of the test organisms to the test chambers marked the initiation of the toxicity tests. Ten organisms were randomly introduced into each replicate beaker.

The test chambers were maintained in a water bath at 23°C, with a 16-hour light/8-hour dark photoperiod. The *H. azteca* were fed 1 ml of YCT (a suspension of yeast, ground cereal leaves, and trout chow) per replicate daily. The overlying water in the exposure chambers was renewed twice daily using a water delivery system (Zumwalt et al. 1994). Fresh overlying water was slowly added to each replicate, allowing the water already in the beaker to flow out through a notch cut into the top of the beaker. The notch was sealed with fine mesh screen to prevent any organisms from being flushed out of the test chamber.

For the amphipod toxicity testing, water quality parameters of temperature, pH, dissolved oxygen, and conductivity were recorded daily on the overlying water in one replicate of each sediment. Composite samples of the overlying water of each sediment were also analyzed for alkalinity, hardness, conductivity and ammonia at test initiation and test termination.

At the end of the 28-day (*H. azteca*) exposure period, the surviving organisms from each replicate were retrieved from the sediment. The number of surviving organisms from each replicate was recorded. The *H. azteca* were examined under a microscope to determine the sex of the organism and the presence or absence of eggs in the females. The surviving *H. azteca* from each replicate were then placed in a dried, pre-weighed aluminum pan and placed in a drying oven at 100°C for 24 hours. The pans were then removed from the oven, placed in a desiccator to cool, and weighed. The dry weight of the surviving organisms in each replicate was determined by subtracting the weight of the empty pan from the weight of the pan plus dried organisms. The mean dry weight per organism was obtained by dividing the total organism dry weight per replicate by the number of surviving organisms per replicate.

The results of the *H. azteca* toxicity tests were statistically analyzed according to US EPA guidance (US EPA 2000) to determine if the sediments were significantly different ($p=0.05$) from the control sediment with respect to survival, growth, or reproduction. If the data were normally distributed, then a t-Test was performed to detect statistically significant differences between a test sediment and the control sediment. If the data distribution was non-normal, then a Wilcoxon Two-Sample Test was used to compare the group means. Shapiro-Wilk's Test was used to

determine if the data were normally distributed, and the F-Test was used to test for homogeneity of variance.

Tables 4 and 5 presents the water quality summary and test results from the *H. azteca* toxicity testing. Copies of the original data sheets and statistical analyses for the *H. azteca* toxicity test are provided in Attachment III.

2.6 REFERENCE TOXICANT TESTS

In conformance with EA's quality assurance/quality control program, reference toxicant tests were performed on the in-house cultured *H. azteca*, and on the acquired lot of *C. riparius*. The amphipods were exposed to the reference toxicant copper sulfate (CuSO_4) in a graded concentration series to determine the 96-hour median lethal concentration (LC_{50}). The midges were exposed to sodium dodecyl sulfate (SDS) to determine the 48-hour LC_{50} . The results of the reference toxicant tests were compared to EA's established control chart limits according to US EPA methodology (US EPA 2002). Reference toxicant test data are presented in Table 6.

2.7 ARCHIVES

Original data sheets, records, memoranda, notes, and computer printouts are archived at EA's Office in Hunt Valley, Maryland. These data will be retained for a period of 5 years unless a longer period of time is requested.

3. RESULTS AND DISCUSSION

3.1 *Chironomus riparius* SEDIMENT TOXICITY TESTS

The results of the *C. riparius* sediment toxicity tests comply with current NELAC standards. A summary of water quality measurements recorded during the test is included in Table 2, and test results are presented in Table 3. After 10 days of exposure, mean survival in all but one sediment sample (SW15-SB31-SURF, AT5-286, 64 percent survival) was significantly less ($p=0.05$) than control (73 percent survival). The remaining site sediments had 34 to 45 percent survival and were all significantly different than the control. Mean ash-free dry weight in the sediment samples ranged from 0.474 to 0.544 mg/organism, and were not significantly different from the control, which had mean ash-free dry weight of 0.484 mg/organism.

3.2 *Hyaella azteca* SEDIMENT TOXICITY TESTS

The results of the *H. azteca* sediment toxicity tests comply with current NELAC standards. Water quality measurements taken during the test are presented in Table 4, and test results are summarized in Table 5. The survival, growth and fecundity of *H. azteca* exposed to the six site sediments were statistically compared to organisms exposed to the lab control sediment. Site sediment sample SW15-SLB02-SURF (AT5-282) was statistically different ($p=0.05$) from the control sample based on survival (79 versus 96 percent survival). Survival in the remaining site sediment samples ranged from 95 to 99 percent and were not significantly different than the control. Mean dry weight in the SW15-SLB05-SURF (AT5-279) and SW15-SLB14-SURF (AT5-280) was 0.275 and 0.251 mg/organism, respectively, and were significantly different than the control (0.328 mg/organism). The remaining site sediments had mean dry weights ranging from 0.327 to 0.617 mg/organism and were not significantly different than the control. Fecundity in SW15-SLB14-SURF (AT5-280) was 55 percent females with eggs, which was significantly different than the 73 percent females with eggs in the control. The remaining site sediments had 60 to 84 percent females with eggs, and were not significantly different than the control.

3.3 REFERENCE TOXICANT TESTS

The results of the reference toxicant testing (Table 6) indicated that the in-house cultured *H. azteca* and the acquired lots of *C. riparius* were of acceptable quality, and the test results were within acceptable control chart limits. An insufficient number of reference toxicant tests have been conducted with *C. riparius* to determine acceptable control chart limits ($N \leq 5$), however, the results of the reference toxicant test conducted for the organisms in this study were within range of the previous reference toxicant test conducted with this species. Additionally, the results of the *C. riparius* reference toxicant testing fell within the acceptable control chart limits for the alternate midge testing species (*C. dilutus*).

4. REFERENCES

- EA. 2013. EA Ecotoxicology Laboratory Quality Assurance and Standard Operating Procedures Manual. EA Manual ATS-102. Internal document prepared by EA's Ecotoxicology Laboratory, EA Engineering, Science, and Technology, Inc., Hunt Valley, Maryland.
- US EPA. 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fifth Edition. EPA-821-R-02-012. U.S. Environmental Protection Agency, Office of Water, Washington, D.C.
- US EPA. 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates. Second Edition. EPA/600/R-99/064. U.S. Environmental Protection Agency, Office of Research and Development, Duluth, Minnesota.
- Zumwalt, D.C., F.J. Dwyer, I.E. Greer, and C.G. Ingersoll. 1994. A water-renewal system that accurately delivers small volumes of water to exposure chamber. Environmental Toxicology and Chemistry. 13:1311-1314.

TABLE 1 COLLECTION AND RECEIPT INFORMATION FOR SEDIMENT
SAMPLES FROM SUPERIOR WATERFRONT CHARACTERIZATION –
ST. LOUIS RIVER AREA AND BAY AREA OF CONCERN, MICHIGAN

<u>Sediment Sample Identification</u>	<u>EA Accession Number</u>	<u>Collection Date</u>	<u>Receipt Time and Date</u>	<u>Receipt Temperature</u>
SW15-SLB05-SURF	AT5-279	8 July 2015	1019, 10 July 2015	4.8 °C
SW15-SLB14-SURF	AT5-280	7 July 2015	1019, 10 July 2015	5.1 °C
SW15-SB04-SURF	AT5-281	10 July 2015	0930, 13 July 2015	7.7 °C
SW15-SLB02-SURF	AT5-282	9 July 2015	0930, 13 July 2015	5.5 °C
SW15-SB19-SURF	AT5-285	16 July 2015	1014, 17 July 2015	5.5 °C
SW15-SB31-SURF	AT5-286	14 July 2015	1014, 17 July 2015	5.4 °C

TABLE 2 WATER QUALITY PARAMETERS MEASURED DURING *Chironomous riparius* 10-DAY TOXICITY TESTING WITH SEDIMENTS FROM SUPERIOR WATERFRONT CHARACTERIZATION – ST. LOUIS RIVER AREA AND BAY AREA OF CONCERN, MICHIGAN

Test Species: *Chironomous riparius* (midge)
Sample Matrix: Sediments
Test Number: TN-15-353

Sample Identification: Accession Number: Water Quality Parameters		Laboratory Control AT4-590	SW15-SLB05-SURF AT5-279	SW15-SLB14-SURF AT5-280	SW15-SB04-SURF AT5-281	SW15-SLB02-SURF AT5-282	SW15-SB19-SURF AT5-285	SW15-SB31-SURF AT5-286
Temperature (°C):		22.0 – 23.5	22.0 – 23.0	22.0 – 23.0	22.0 – 22.8	22.0 – 22.9	22.0 – 22.8	22.0 – 22.8
pH:		7.2 – 8.1	7.2 – 8.0	7.2 – 7.9	7.2 – 7.9	7.2 – 7.8	6.9 – 7.8	7.1 – 7.8
Dissolved Oxygen: (mg/L)		5.6 – 8.0	4.7 – 6.9	4.0 – 6.7	4.7 – 7.7	4.8 – 7.3	5.0 – 6.9	5.6 – 7.6
Conductivity: (µS/cm)		340 – 360	342 – 363	347 – 371	347 – 394	349 – 380	343 – 367	340 – 361
Alkalinity: (mg/L CaCO ₃)	Day 0	48	66	64	100	72	72	54
	Day 10	52	68	78	74	74	72	64
Hardness: (mg/L CaCO ₃)	Day 0	92	100	128	112	104	100	92
	Day 10	88	100	96	96	112	100	76
Conductivity: (µS/cm)	Day 0	315	346	361	405	372	368	330
	Day 10	318	345	356	354	350	356	341
Ammonia (mg/L NH ₃ -N)	Day 0	2.28	1.15	0.69	2.13	0.90	2.91	0.76
	Day 10	0.67	1.55	2.00	1.70	1.25	1.73	1.27

TABLE 3 RESULTS OF *Chironomous riparius* 10-DAY TOXICITY TESTING ON SEDIMENTS FROM SUPERIOR WATERFRONT CHARACTERIZATION – ST. LOUIS RIVER AREA AND BAY AREA OF CONCERN, MICHIGAN

Test Species: *Chironomous riparius* (midge)
 Sample Matrix: Sediments
 EA Test Number: TN-15-353
 Test Initiation: 29 August 2015
 Test Termination: 8 September 2015

Sample Identification	EA Accession Number	10-Day Survival (percent)	Ash Free Mean Dry Weight as mg/Organism (\pm SD)
Laboratory Control	AT4-590	73	0.484 (\pm 0.067)
SW15-SLB05-SURF	AT5-279	43 ^(a)	0.474 (\pm 0.101)
SW15-SLB14-SURF	AT5-280	41 ^(a)	0.544 (\pm 0.072)
SW15-SB04-SURF	AT5-281	45 ^(a)	0.499 (\pm 0.097)
SW15-SLB02-SURF	AT5-282	34 ^(a)	0.506 (\pm 0.116)
SW15-SB19-SURF	AT5-285	40 ^(a)	0.510 (\pm 0.119)
SW15-SB31-SURF	AT5-286	64	0.544 (\pm 0.133)

(a) Significantly different ($p=0.05$) from laboratory control.

TABLE 4 WATER QUALITY PARAMETERS MEASURED DURING *Hyalella azteca* 28-DAY TOXICITY TESTING WITH SEDIMENTS FROM SUPERIOR WATERFRONT CHARACTERIZATION – ST. LOUIS RIVER AREA AND BAY AREA OF CONCERN, MICHIGAN

Test Species: *Hyalella azteca* (amphipod)
Sample Matrix: Sediments
Test Number: TN-15-308

Sample Identification: Accession Number: Water Quality Parameters		Laboratory Control AT4-590	SW15-SLB05-SURF AT5-279	SW15-SLB14-SURF AT5-280	SW15-SB04-SURF AT5-281	SW15-SB03-SURF AT5-282	SW15-SB19-SURF AT5-285	SW15-SB31-SURF AT5-286
Temperature (°C):		22.0 – 24.0	22.0 – 23.9	22.0 – 23.9	22.0 – 23.8	22.0 – 23.8	22.0 – 23.7	22.0 – 23.7
pH:		7.3 – 8.2	7.3 – 8.1	7.3 – 8.1	7.3 – 8.0	7.3 – 8.0	7.3 – 7.9	7.3 – 7.9
Dissolved Oxygen: (mg/L)		4.9 – 7.9	4.5 – 7.0	4.3 – 6.8	5.3 – 7.6	5.1 – 7.1	4.2 – 7.1	5.1 – 7.4
Conductivity: (µS/cm)		320 – 359	327 – 379	333 – 374	338 – 387	333 – 374	324 – 364	321 – 370
Alkalinity: (mg/L CaCO ₃)	Day 0	40	60	72	84	76	78	54
	Day 28	62	74	72	76	74	66	66
Hardness: (mg/L CaCO ₃)	Day 0	92	92	104	108	100	92	84
	Day 28	108	120	108	124	116	108	116
Conductivity: (µS/cm)	Day 0	299	333	348	389	365	352	318
	Day 28	348	372	369	364	358	363	330
Ammonia (mg/L NH ₃ -N)	Day 0	2.32	1.04	0.99	1.61	1.12	3.21	0.86
	Day 28	<0.10	0.10	0.19	<0.10	0.17	0.14	0.12

TABLE 5 RESULTS OF *Hyalella azteca* 28-DAY TOXICITY TESTING ON SEDIMENTS FROM SUPERIOR WATERFRONT CHARACTERIZATION – ST. LOUIS RIVER AREA AND BAY AREA OF CONCERN, MICHIGAN

Test Species: *Hyalella azteca* (amphipod)
Sample Matrix: Sediments
EA Test Number: TN-15-308
Test Initiation: 6 August 2015
Test Termination: 3 September 2015

Sample Identification	EA Accession Number	28-Day Survival (percent)	Mean Dry Weight as mg/Organism (\pm SD)	Mean Fecundity as Percent Females with Eggs
Laboratory Control	AT4-590	96	0.328 (\pm 0.039)	73
SW15-SLB05-SURF	AT5-279	98	0.275 (\pm 0.032) ^(a)	69
SW15-SLB14-SURF	AT5-280	95	0.251 (\pm 0.027) ^(a)	55 ^(a)
SW15-SB04-SURF	AT5-281	99	0.617 (\pm 0.092)	89
SW15-SLB02-SURF	AT5-282	79 ^(a)	0.346 (\pm 0.082)	60
SW15-SB19-SURF	AT5-285	96	0.601 (\pm 0.090)	84
SW15-SB31-SURF	AT5-286	99	0.327 (\pm 0.052)	64

(a) Significantly different (p=0.05) from laboratory control.

TABLE 6 RESULTS OF REFERENCE TOXICANT TESTING

Test Species	Reference Toxicant	EA Test Number	Test Result	Acceptable Control Chart Limits
<i>Hyalella azteca</i> (amphipod)	Copper sulfate (CuSO ₄)	RT-15-134	96-Hour LC50: 164 µg/L Cu	0 – 341 µg/L Cu
<i>Chironomus riparius</i> (midge)	Sodium dodecyl sulfate (SDS)	RT-15-114	48-Hour LC50: 42 mg/L SDS	9 – 75 mg/L SDS ^(a)

(a) An insufficient number of reference toxicant tests have been conducted with *C. riparius* (N=<5) to determine acceptable control chart limits; however, the results of this test were comparable to previous reference toxicant tests conducted with this species. The results reported are for another midge test species *C. dilutus* (formerly *tentans*).

ATTACHMENT I

Chain-of-Custody Record
(4 pages)

ATTACHMENT II

Data Sheets and Statistical Analyses
from *Chironomus riparius* Sediment Toxicity Test
(31 pages)

ATTACHMENT III

Data Sheets and Statistical Analyses
from *Hyalella azteca* Sediment Toxicity Test
(42 pages)

ATTACHMENT IV

Report Quality Assurance Record
(2 pages)